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Antibiotic combination therapy can select for broad-spectrum multidrug resistance in *Pseudomonas aeruginosa*

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ABSTRACT

Combination therapy with several antibiotics is one strategy that has been applied in order to limit the spread of antimicrobial resistance. We compared the de novo evolution of resistance during combination therapy with the β-lactam ceftazidime and the fluoroquinolone ciprofloxacin with the resistance evolved after single-drug exposure. Combination therapy selected for mutants that displayed broad-spectrum resistance, and a major resistance mechanism was mutational inactivation of the repressor gene *mexR* that regulates the multidrug efflux operon *mexAB-oprM*. Deregulation of this operon led to a broad-spectrum resistance phenotype that decreased susceptibility to the combination of drugs applied during selection as well as to unrelated antibiotic classes. Mutants isolated after single-drug exposure displayed narrow-spectrum resistance and carried mutations in the MexCD–OprJ efflux pump regulator gene *nfxB* conferring ciprofloxacin resistance, or in the gene encoding the non-essential penicillin-binding protein DacB conferring ceftazidime resistance. Reconstruction of resistance mutations by allelic replacement and in vitro fitness assays revealed that in contrast to single antibiotic use, combination therapy consistently selected for mutants with enhanced fitness expressing broad-spectrum resistance mechanisms.

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1. Introduction

Combination therapy with two or more drugs is the standard treatment for infections with human immunodeficiency virus (HIV), *Plasmodium falciparum*, *Mycobacterium tuberculosis* and, in cystic fibrosis (CF) patients, *Pseudomonas aeruginosa* [1–4]. There are multiple rationales for combination therapy. The clinical considerations include therapeutically covering the spectrum of potential pathogens during polymicrobial infections or in acute infections for which the responsible micro-organism or resistance profile of the pathogen is unknown [5]. Using different classes of antimicrobial drugs in combination might result in synergistic antibiotic interactions that enhance the inhibitory effect [5]. None the less, the general benefits of combination therapy compared with single or sequential administration of antibiotics for treating bacterial infections have been difficult to conclusively demonstrate

[4]. Combination treatment might even have negative effects in infections with Gram-negative bacteria owing to increased treatment costs and increased risk of adverse side effects. However, since combination therapy could potentially lower the rate of de novo antibiotic resistance evolution, it is considered a potential resistance management strategy [4].

Theoretically, the probability of mutations conferring resistance to two agents simultaneously is lower than the probability of mutations conferring resistance to a single agent. However, this probability applies only if resistance mutations occur independently and drugs are administered simultaneously for enhanced effect. Considering the pharmacodynamic and pharmacokinetic complexity of multidrug treatment, reaching synchronised, therapeutically inhibitory concentrations at the site of infection in the clinical setting is problematic [2,6]. General resistance phenotypes caused by biofilm growth or persister state, or the potential selection for broad-spectrum resistance mechanisms activated via one-step mutational events confounds predictions about the potential overall benefit of combination therapy over monotherapy as a tool for controlling the spread of antibiotic resistance. We propose the following model by which bacterial populations acquire

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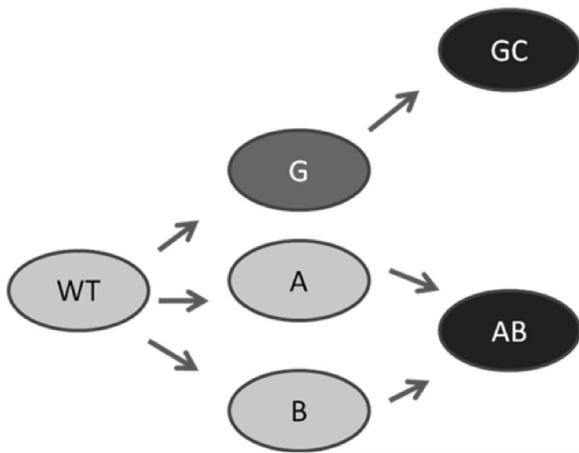


Fig. 1. Pathways for selecting general resistance mechanisms. A mutation or state that gives a general resistance phenotype has a selection advantage over specific resistance mutations when two drugs are present. 'A' (*dacB*) and 'B' (*nfxB*) confer resistance to drug 'a' (ceftazidime) and drug 'b' (ciprofloxacin), respectively; 'G' (*mexR* or unknown) confers a general resistance phenotype; 'C' represents a compensatory event that either increases resistance or reduces the fitness cost of G. The data from this study showed that G (*mexR* or unknown) can occur at the same frequency as A (*dacB*) or B (*nfxB*), increasing the probability of broad-spectrum resistance when two drugs are present. WT, wild-type.

broad-spectrum resistance during combination therapy. Resistance to antibiotics 'a' and 'b' is conferred by the resistance mutations 'A' and 'B', either through the acquisition of first A and then B, or first B and then A (Fig. 1). However, we hypothesise that in the presence of both drugs, a mutation or state that results in a general resistance phenotype ('G') towards a and b simultaneously confers a selective advantage compared with the specific resistance mutations (A or B) when the two drugs are present simultaneously. The G state does not necessarily need to provide a survival advantage compared with an A + B double mutant, but will have a fitness advantage compared with the A or B single mutant when both drugs are present. Moreover, the G state will have the advantage of requiring fewer additional mutations to reach full resistance and will therefore change the resistance evolution trajectory by potentiating the evolution of novel resistance traits, either acquisition of the specific resistance mutations a and b, which requires multiple mutations, or acquisition of an additional compensatory mutation 'c' that enhances G to levels of resistance comparable with resistance provided by A and B (Fig. 1). To understand the consequences

of antibiotic combination therapy, we investigated the spectrum of resistance mutations that were selected during single-drug, sequential drug and combination therapy. In vitro experiments were performed challenging the Gram-negative human pathogen *P. aeruginosa* with a β -lactam drug ceftazidime that targets cell-wall synthesis, primarily penicillin-binding protein 3 (PBP3), and a fluoroquinolone ciprofloxacin that disrupts DNA replication by inhibiting the enzymes topoisomerase II and topoisomerase IV [7,8]. The antibiotics tested in the present study are used in the treatment of CF patients with *P. aeruginosa* lung infections, who are sometimes simultaneously treated with both drugs [3,9]. The molecular mechanisms underlying the resistance phenotypes were identified using whole-genome sequencing and allelic replacement. Each therapy selected for different resistance mechanisms, with combination therapy resulting in broad-spectrum resistance whereas antibiotic monotherapy selected for narrow-spectrum resistance. These results have important implications for combination therapy as a clinical tool for antibiotic resistance management.

2. Materials and methods

2.1. Strains, media, susceptibility testing and mutant selection

Derivatives of *P. aeruginosa* strain PAO1 were used in this study [10]. A complete list of strains and plasmids is presented in Table 1. All strains were grown at 37 °C in Luria–Bertani (LB) broth with aeration at 200 rpm with or without ciprofloxacin (Bayer HealthCare AG, Leverkusen, Germany) and/or ceftazidime (Sigma-Aldrich, St Louis, MO). Minimum inhibitory concentrations (MICs) were determined for ceftazidime, ciprofloxacin and meropenem using Etest strips (bioMérieux S.A., Marcy-l'Étoile, France) on LB agar plates at 37 °C for 22 h according to the manufacturer's guidelines. Etest MICs for combinations of ciprofloxacin and ceftazidime were determined according to the manufacturer's guidelines (Etest application sheet EAS 023; AB BIODISK, bioMérieux S.A.). Selection of resistant mutants was on LB agar plates containing antimicrobials at 5× MIC for PAO1 (4 mg/L ceftazidime and 1 mg/L ciprofloxacin for single-drug therapy, and 1.5 mg/L ceftazidime + 0.2 mg/L ciprofloxacin for combination therapy). Single-drug selections were also performed at 1.5 mg/L ceftazidime and 0.2 mg/L ciprofloxacin. Mutation frequencies were calculated as antibiotic-resistant CFU divided by total CFU determined on antibiotic-free Luria agar plates.

Table 1
Additional strains used in this study.

Strain	Relevant genotype/phenotype	Selecting agent (g/mL)	Source/reference
PAO1	<i>Pseudomonas aeruginosa</i> wild-type		[10]
MV146	<i>mexR</i> (G188A)		This study
MV148	<i>dacB</i> (G211A)		This study
MV176	<i>dacB</i> (G211A) + <i>nfxB</i> (C65T)		This study
MV182	<i>nfxB</i> (C65T)		This study
MV124		CIP 1	This study
MV125		CIP 1	This study
MV122		CAZ 4	This study
MV123		CAZ 4	This study
MV237		CIP 0.2 + CAZ 1.5	This study
MV132		CIP 0.2 + CAZ 1.5	This study
MV155		CIP 0.2 + CAZ 1.5	This study
MV152		CIP 0.2 + CAZ 1.5	This study
MV127		CAZ 4 → CIP 1	This study
MV128		CAZ 4 → CIP 1	This study
MV129		CAZ 4 → CIP 1	This study
CC118 λ . <i>pir</i>	<i>Escherichia coli</i> lysogenised with λ . <i>pir</i> phage		[11]
pRK600	RK2-Mob ⁺ RK2-Tra ⁺ , Cm ^r		[12]
pNJ1	<i>sacB</i> , Tet ^r , R6 K origin		[16]

2.2. Genome sequencing and mutation analysis

Genome sequencing of isolates was performed using an Illumina HiSeq™ 2000 machine (Illumina Inc., San Diego, CA) generating 100-bp paired-end reads as previously described [13] to an average genomic coverage of at least 74-fold (range 74- to 135-fold). Mutations were identified as previously described [14].

2.3. Mutant construction

Mutations from antibiotic-selected resistant strains were transferred by allelic replacement into PAO1 and triparental mating using the helper strain *Escherichia coli* CC118 λ pir/pRK600 as described previously [15]. Primers for mutant construction are presented in Supplementary Table S1. Constructs with relevant gene products were incorporated into pNJ1 plasmid [16] and were used to transform the helper strain *E. coli* CC118 λ pir. Selection of merodiploids was performed by plating the conjugation mixture on *Pseudomonas* isolation agar (Sigma-Aldrich) supplemented with 50 mg/L tetracycline as described previously [17]. Confirmation of incorporation of mutations was performed by PCR with mutation-specific primers [17].

2.4. Quantitation of resistance gene expressions by quantitative PCR (qPCR)

RNA was isolated using an SV RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA was converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) with an RNase inhibitor. The cDNA was used as template for real-time qPCR using the primers in Supplementary Table S2 and Maxima SYBR Green/ROX qPCR Master Mix (Applied Biosystems). PCR products were detected using an MX3000P qPCR System (Agilent Technologies, Santa Clara, CA).

2.5. Fitness measurements

Three assays were used to estimate fitness. (i) The growth rate of bacteria grown in LB at 37 °C with optical density at 600 nm measured over time using a Bioscreen C reader (Labsystems Oy, Helsinki, Finland) and 100-well honeycomb plates with 300 μ L/well of overnight culture diluted to a starting concentration of 10⁶ CFU/mL. The relative fitness of strains was calculated as the ratio of the mutant growth rate to that of the wild-type (WT). (ii) Stationary phase CFU were determined after 24 h of growth at 200 rpm in 5 mL of LB medium in 50-mL polypropylene centrifuge tubes, with a starting inoculum of ca. 10⁷ CFU/mL. (iii) Competition experiments were used to determine relative competitive fitness (W) after 24 h for resistant strains isogenic to WT, which was calculated using the formula [18]: $W = \ln(RF/RI) / \ln(SF/SI)$, where RI and SI are the CFU of resistant and susceptible cells at the start of the competition assay, respectively, and RF and SF are the number of resistant and susceptible cells at the endpoint of the competition assay. For the competition assay, overnight cultures of isogenic resistant and WT cells were diluted to ca. 10⁷ CFU/mL, were mixed at 1:1 and were co-cultured for eight to nine generations before reaching stationary phase (using the same culturing conditions as for determining CFU described above).

Endpoint CFU (competition ratio) between resistant and WT cells was determined by spreading suitable dilutions on Luria agar plates with or without antibiotic concentrations appropriate for selecting the specific resistance. For each condition assayed, three to five biological replicates were used. For statistical analysis, one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test as post-hoc analysis was used. All statistical

calculations were performed using GraphPad Prism v.5.00 for Windows (GraphPad Software, San Diego, CA).

2.6. Nucleotide sequence accession numbers

Sequence reads from all isolates are deposited in the European Nucleotide Archive under study accession no. PRJEB8026.

3. Results

3.1. High resistance frequency with the ciprofloxacin + ceftazidime antibiotic combination

Combinations of antibiotics from four drug classes targeting distinct cellular functions were tested for synergistic effects against the reference strain *P. aeruginosa* PAO1 [10]. Ciprofloxacin was used in four combinations, paired with the third-generation cephalosporin ceftazidime, the carbapenems meropenem or imipenem, or the aminoglycoside tobramycin. Of the four combinations investigated, three showed an indifferent fractional inhibitory concentration index (FICI) using an Etest-based fractional inhibitory concentration assay [19]. Only the ceftazidime+ciprofloxacin combination had an additive effect (FICI=0.96). The ceftazidime+ciprofloxacin and ceftazidime+tobramycin combinations were selected for further investigations on resistance development. The resistance frequencies towards ciprofloxacin, ceftazidime and tobramycin were determined at 5 \times MIC for single, sequential and combination therapy. Owing to the additive effect from the tested antibiotic combinations, the absolute concentrations had to be adjusted in order to maintain a relative MIC exposure between the single and combination therapies. The fluctuation tests with single, sequential and combination therapy gave the following frequencies: monotherapy, 4 \times 10⁻⁸ for ciprofloxacin and 2 \times 10⁻⁷ for ceftazidime; sequential treatment, 5 \times 10⁻⁸ using both the single ciprofloxacin-resistant mutant (MV124) and the ceftazidime-resistant mutant (MV122) (Table 2). The resistance frequency of the ceftazidime+ciprofloxacin combination was 4 \times 10⁻⁸, showing that the selection for resistant mutants could neither be prevented nor substantially decreased with the combination of antibiotics. When the concentration was 10 \times MIC (ciprofloxacin 0.5 mg/L and ceftazidime 4 mg/L) no mutants appeared. The ceftazidime+tobramycin combination could, however, prevent the emergence of resistant mutants at 5 \times MIC, therefore tobramycin monotherapy and sequential therapy were not further investigated.

3.2. Combination therapy selects for broad-spectrum resistance mechanisms

From each treatment procedure, a minimum of 20 independent resistant colonies were selected and resistance to ceftazidime and ciprofloxacin was investigated. Resistance to meropenem, which

Table 2
Frequencies of antibiotic resistance mutations for strains selected on CAZ, CIP, the CAZ+CIP combination, or sequentially selected as CAZ \rightarrow CIP or CIP \rightarrow CAZ.

Antimicrobial agent (mg/L ^a)	Strain		
	PAO1	MV122 ^b	MV124 ^c
CAZ 4	1.9 \times 10 ⁻⁷	N/D	5.3 \times 10 ⁻⁸
CIP 1	3.7 \times 10 ⁻⁸	4.7 \times 10 ⁻⁸	N/D
CAZ 1.5 + CIP 0.2	3.9 \times 10 ⁻⁸	N/D	N/D

CAZ, ceftazidime; CIP, ciprofloxacin; N/D, not determined; MIC, minimum inhibitory concentration.

^a Selection concentrations were 5 \times MIC of the sensitive PAO1 strain.

^b CAZ-resistant strain.

^c CIP-resistant strain.

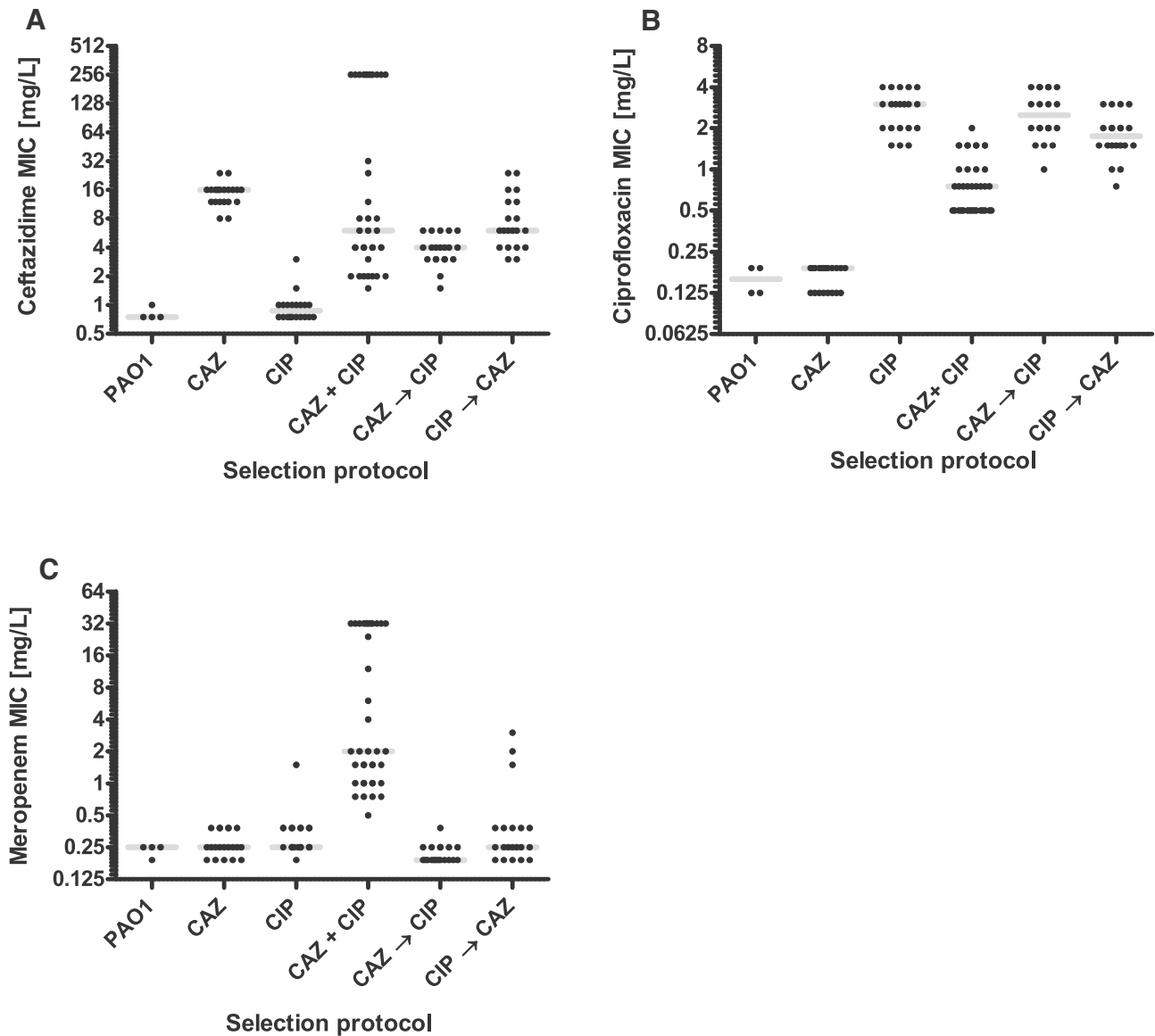


Fig. 2. Strains selected on a combination of ciprofloxacin + ceftazidime are also resistant to meropenem. Resistance to (A) ceftazidime, (B) ciprofloxacin and (C) meropenem. The x-axis designates the different selection protocols used. PAO1 designates the parental sensitive strain; CAZ designates selection on ceftazidime, CIP designates selection on ciprofloxacin; CAZ + CIP designates selection on both antibiotics; and CAZ → CIP or CIP → CAZ designate sequential selection. Light grey lines show the median with range. MIC, minimum inhibitory concentration.

was not used in any of the selections, was examined to assess broad-spectrum resistance (Fig. 2A–C; Supplementary Table S3). The median resistance of the ceftazidime-selected strains was increased 20-fold compared with the parental PAO1 strain (Fig. 2A). The ceftazidime-selected strains showed no changes in susceptibility to ciprofloxacin or meropenem (Fig. 2B and C) (Supplementary Table S3). The median MIC of ciprofloxacin-selected strains was increased 20-fold compared with the parental PAO1, and 19 of 20 ciprofloxacin-selected strains showed no change in resistance to ceftazidime or meropenem. Of 20 ciprofloxacin-selected strains, 1 had reduced susceptibility to both β -lactams (Fig. 2A and C) (Supplementary Table S3). However, all strains selected with the ceftazidime + ciprofloxacin combination displayed reduced susceptibility not only to ceftazidime and ciprofloxacin but also towards meropenem (Fig. 2A and C) (Supplementary Table S3). The median MIC for ceftazidime + ciprofloxacin-selected strains was 6 mg/L to ceftazidime and 0.75 mg/L to ciprofloxacin. The median susceptibility of ceftazidime + ciprofloxacin-selected strains to

meropenem was 6-fold less than PAO1 (Fig. 2; Supplementary Table S3). Of 40 sequentially selected strains, 3 exhibited reduced susceptibility to meropenem, with resistance similar to ceftazidime + ciprofloxacin-selected strains; the other 37 strains exhibited decreased susceptibility only to ceftazidime and ciprofloxacin (Fig. 2; Supplementary Table S3).

To achieve a 5 \times MIC adjusted selection concentration for the combination and the single selection, the absolute concentrations of ciprofloxacin and ceftazidime were lower in the combination than for the single selections. Therefore, five independent mutants were selected on low-level ciprofloxacin concentration (0.2 mg/L) and ceftazidime (1.5 mg/L) corresponding to 1–2 \times MIC of PAO1.

Two of five strains ciprofloxacin-selected strains exhibited a general resistance phenotype with reduced susceptibility towards ciprofloxacin, ceftazidime and meropenem (Supplementary Table S3), whilst three strains showed specific resistance towards ciprofloxacin. Two ceftazidime-selected strains showed only resistance to ceftazidime, to similar levels as the strains selected on

Table 3
Genome sequencing of resistant mutants selected on CAZ, CIP, the CAZ + CIP combination, or sequentially selected CAZ → CIP.

Strain	Selecting agent (mg/L)	Mutation			Amino acid change
		Gene	Type	Position ^a	
MV124	CIP 1	<i>nfxB</i>	Insertion	77(1 bp)78	
MV125	CIP 1	<i>nfxB</i>	Missense	C65T	P22L
MV122	CAZ 4	<i>dacB</i>	Deletion	1280(15 bp)1294	
MV123	CAZ 4	<i>dacB</i>	Missense	G211A	A71T
MV237	CIP 0.2 + CAZ 1.5	<i>mexR</i>	Deletion	145(3 bp)147	
MV132	CIP 0.2 + CAZ 1.5	<i>mexR</i>	Insertion	321(1 bp)322	Stop
MV155	CIP 0.2 + CAZ 1.5	<i>mexR</i>	Missense	G188A	R63H
MV127 ^b	CAZ 4 → CIP 1	<i>nfxB</i>	Deletion	85(11 bp)95	
		<i>dacB</i>	Deletion	1280(15 bp)1294	
MV128 ^b	CAZ 4 → CIP 1	<i>nfxB</i>	Nonsense	C391T	Q131
		<i>dacB</i>	Deletion	1280(15 bp)1294	
MV129 ^b	CAZ 4 → CIP 1	<i>nfxB</i>	Missense	G541C	A181P
		<i>dacB</i>	Deletion	1280(15 bp)1294	

CAZ, ceftazidime; CIP, ciprofloxacin.

^a Position of mutational change relative ATG start codon.

^b MV122 used for sequential selection.

ceftazidime at 5 × MIC (Supplementary Table S3), and three strains exhibited a general resistance phenotype with reduced susceptibility to ceftazidime, ciprofloxacin and meropenem.

3.3. Mutations in *mexR* confer broad-spectrum resistance via derepression of MexAB-mediated efflux

Whole-genome sequencing was performed on 10 selected strains to identify potential resistance mutations (Table 3). In all sequenced strains selected on ciprofloxacin, *nfxB* mutations were identified. NfxB is a transcriptional regulator that represses expression of the *mexCD–oprJ* genes, which encode the drug efflux pump MexCD–OprJ. This is a common mechanism of resistance to ciprofloxacin in clinical isolates [20]. The two strains selected on ceftazidime had mutations in *dacB*, which encodes the non-essential PBP4 that is involved in ceftazidime resistance through regulation of AmpC expression [21]. Mutations in *mexR* were identified as potential resistance-conferring mutations in the sequenced strains selected on ceftazidime + ciprofloxacin. The *mexR* gene encodes the transcriptional regulator MexR that represses expression of the *mexAB–oprM* genes encoding the efflux pump MexAB–OprM. Overexpression of this efflux pump is associated with broad-spectrum efflux of antimicrobial compounds in clinical *Pseudomonas* isolates [22]. Three isolates from a ceftazidime-resistant mutant (MV122) that was subsequently exposed to ciprofloxacin were sequenced. All contained the original mutation in *dacB* and had acquired additional mutations in *nfxB* (Table 3). To confirm the effect of the mutations as the resistance mechanisms, allelic replacement was used to transfer mutations in *dacB* (G211A), *nfxB* (C65T) and *mexR* (G188A) into the sensitive PAO1 background. No significant differences in resistance levels were observed between constructed and selected strains (Supplementary Table S3). The reconstructed *mexR* mutant had MIC levels above (ceftazidime) and at (ciprofloxacin) the drug concentrations used at the single-drug treatments. A strain with mutations in *dacB* (G211A) and *nfxB* (C65T) was made to reconstruct the sequential acquisition of resistance mutations. The constructed *dacB–nfxB* double mutant showed resistance towards only ceftazidime and ciprofloxacin with no change in susceptibility to meropenem. The *dacB–nfxB* double mutant had resistance to ciprofloxacin (Supplementary Table S3) that was similar to the strain carrying only an *nfxB* mutation but had a reduced MIC towards ceftazidime compared with the single *dacB* mutant.

3.4. *mexA* expression is upregulated in most combination-selected mutants

Mutations in the *dacB* gene influence β -lactam susceptibility by affecting expression of the native β -lactamase gene *ampC* [21]. Real-time reverse transcription qPCR was used to compare *ampC* expression in the reconstructed *dacB* strain (MV148) with PAO1 and 10 ceftazidime-selected strains. The mutation in *dacB* resulted in 110-fold upregulation of *ampC* mRNA in the reconstructed strain. Median expression of the ceftazidime-selected strains was 60-fold higher than the baseline PAO1 expression (Fig. 3; Supplementary Table S3). Ciprofloxacin-selected strains had a median increase in *mexD* expression of 443-fold the PAO1 baseline (Fig. 3). The *dacB–nfxB* double mutant showed significantly ($P < 0.01$) lower *ampC* expression than the *dacB* single mutant. Expression of *mexD* was not significantly affected in the double mutant compared with the *nfxB* mutant. Mutation in *mexR* increased *mexA* mRNA 11.6-fold (Supplementary Table S3) in the reconstructed strain compared with parental PAO1. Of 19 strains selected by ceftazidime + ciprofloxacin combination, 12 had a median 11-fold upregulated *mexA* expression (Fig. 3; Supplementary Table S3). Of the five strains selected at 0.2 mg/L ciprofloxacin, two showed

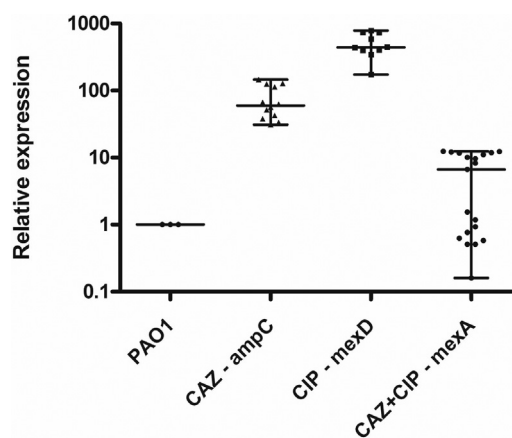


Fig. 3. *mexA* is upregulated in combination antibiotic-selected strains. Relative expression of relevant resistance mechanisms in bacterial isolates selected on CAZ (*ampC*), CIP (*mexD*) or CAZ + CIP combination (*mexA*). Quantification of gene expression was performed by quantitative PCR (qPCR) and was normalised to expression in the antibiotic-sensitive wild-type PAO1. Light grey lines show the median with range. CAZ, ceftazidime; CIP, ciprofloxacin.

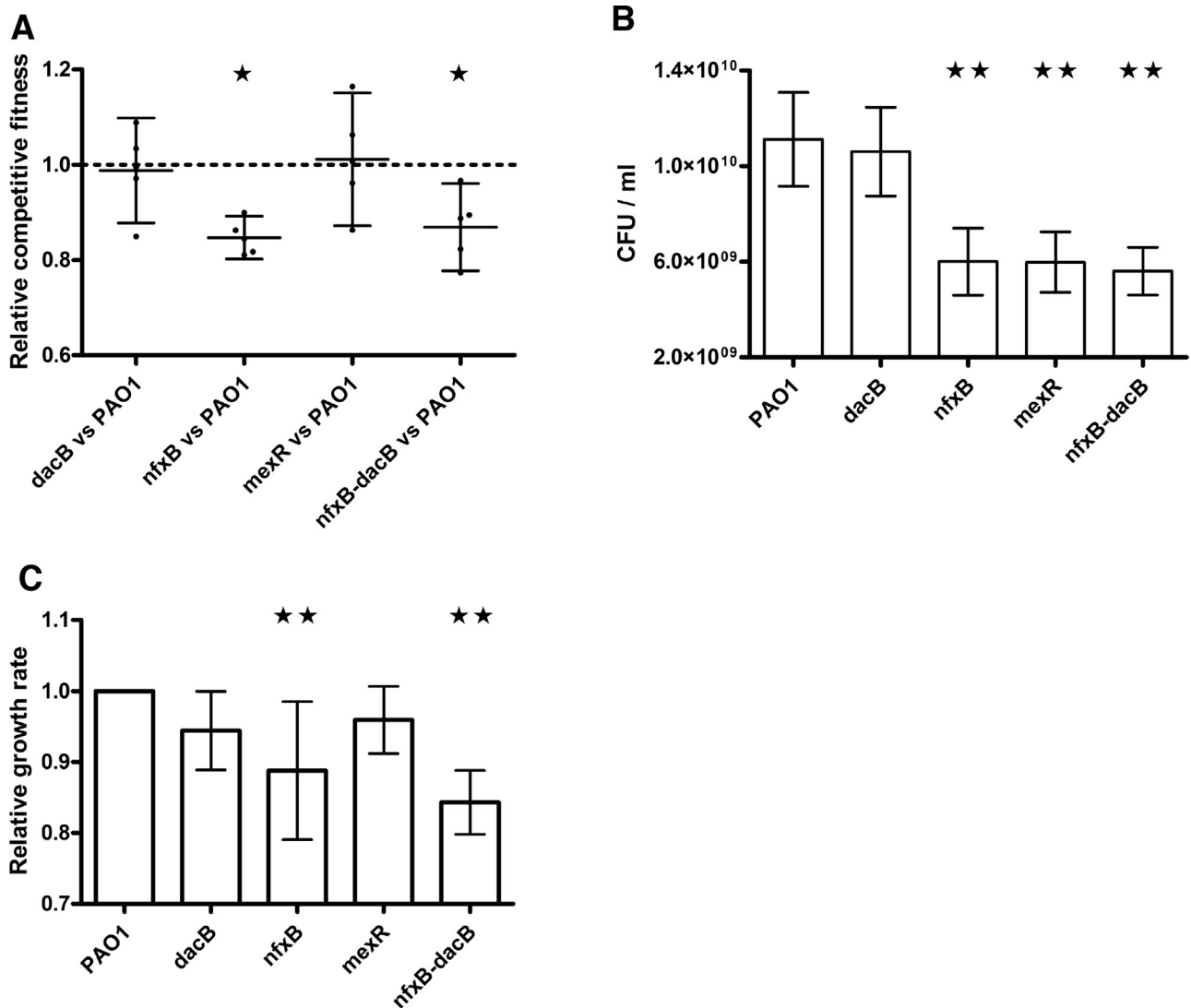


Fig. 4. Mutations in *nfxB* confer the highest fitness cost. Fitness effects of resistance mechanisms were measured. (A) Relative competitive fitness assays in Luria-Bertani (LB) broth comparing the sensitive PAO1 strain in pairwise competition against resistant strains with mutations in the indicated genes. Relative competitive fitness <1 indicates decreased fitness. * $P \leq 0.05$ compared with sensitive PAO1 strain. Shown are means with error bars showing 95% confidence interval. (B) Endpoint CFU after 24 h for cultures grown individually. ** $P \leq 0.01$ compared with the sensitive PAO1 strain. (C) Relative exponential growth rate as the ratio of growth rate of the resistant strain divided by growth rate of the susceptible PAO1 strain. Growth rates were measured in LB and sensitive strains fitness was set to 1.0.

a 2.5–5-fold increased level of *mexA* expression. None of the remaining three had altered expression of *mexD* and the mechanisms of resistance in these strains remain unknown. Three of the five strains selected on 1.5 mg/L ceftazidime had increased expression of *ampC*, whilst the resistance mechanism of the last two was not due to *ampC* overexpression.

3.5. *MexCD* upregulation is associated with a competitive fitness cost

To assess the potential fitness impact of the identified resistance mechanisms, the growth characteristics of the PAO1 strain was compared with four constructed isogenic resistant mutants (*mexR*, *nfxB*, *dacB* and *dacB-nfxB*). The parameters analysed were: (i) exponential growth rate; (ii) CFU in stationary phase after 24 h; and (iii) outcome of pairwise competitions between PAO1 and the four constructed mutants after inoculation at 1:1 and competitive growth for eight to nine generations (Fig. 4A–C). In pairwise competitions in LB broth, a fitness cost was detected only for *nfxB*

and *dacB-nfxB* mutants; no fitness cost was identified for *dacB* and *mexR* mutants (Fig. 4A). The relative competitive fitness of the *nfxB* mutant to PAO1 was 0.85. The fitness cost resulted from a combination of a reduced exponential growth rate of 0.89 relative to PAO1 and a two-fold reduction in CFU in a stationary phase culture. The fitness cost for the *dacB-nfxB* mutant was mainly associated with dysfunctional NfxB because the fitness for the three measured parameters were similar to the *nfxB* mutant with relative competitive fitness of 0.87, relative growth rate of 0.84, and a two-fold reduction in CFU for a stationary phase culture after 24 h (Fig. 4A–C). The ceftazidime resistance mutation in *dacB* had no impact on relative competitive fitness (0.99) compared with PAO1 and no significant difference in growth rate or CFU count for a stationary-phase culture ($P > 0.05$). Similarly, the *mexR* mutant had a relative competitive fitness of 1.01 compared with PAO1 (Fig. 4A) and *mexR* mutants exhibited no significant reduction in exponential growth rate compared with PAO1 (Fig. 4B), but a two-fold reduction in CFU for a stationary-phase culture after 24 h was detected (Fig. 4C).

4. Discussion

Determining the impact of combination therapy on antibiotic resistance evolution and bacterial fitness compared with the effect of monotherapy is important from both an evolutionary and a medical perspective. Combination therapy has been put forward as an alternative to decrease the rate of resistance evolution, but only a few studies have experimentally investigated combination therapy, and the data on the beneficial effects of combination therapy are conflicting [4]. We investigated the frequency of resistance and the spectrum of resistance mechanisms using ceftazidime, ciprofloxacin and tobramycin, three antibiotics commonly used to treat *P. aeruginosa* infections in CF patients. Two antibiotic combinations (ceftazidime + ciprofloxacin and ceftazidime + tobramycin) were assessed to determine changes in resistance evolution trajectories compared with single-agent usage or sequential exposure to the two agents. The ceftazidime + tobramycin combination prevented the selection of resistant mutants at the tested concentration, whilst resistant mutants were selected on the ceftazidime + ciprofloxacin combination. Therefore, resistance evolution to ceftazidime and ciprofloxacin during single, sequential and combination exposure was evaluated. In most resistant isolates, resistance after exposure to a single drug was attributable to increased expression of the cephalosporinase AmpC or the multidrug efflux pump MexCD–OprJ, indicating that mutations in the *dacB* and *nfxB* regulators are responsible for the decreased antibiotic susceptibility (Fig. 2). Inactivating mutations in *dacB* are known to result in AmpC overproduction in clinical and in vitro-selected antibiotic-resistant *P. aeruginosa* isolates [21]. Ceftazidime and ciprofloxacin in combination resulted in resistant mutants at a frequency (3.9×10^{-8}) comparable with the frequency observed after exposure to ciprofloxacin alone (Table 2). In the majority of the strains, resistance was attributable to overexpression of the *mexAB–oprM*-encoding multidrug efflux operon, which facilitates the efflux of the two antibiotics [23]. Not all broad-spectrum resistant mutants exhibited *mexA* overexpression, indicating that at least one additional mechanism can confer broad-spectrum resistance. We were unsuccessful in identifying causative mechanisms in strains that were not overexpressing *mexA*.

We identified clinically relevant levels of resistance towards meropenem in strains isolated after exposure to a combination of ceftazidime and ciprofloxacin (Fig. 2A–C). Meropenem is a carbapenem belonging to a separate class of β -lactam antibiotics that is highly resistant to most β -lactamases [24]. The majority of the resistant strains isolated after exposure to a single drug had a resistance profile that was specific to the agent used for selection; only 1 of 40 investigated strains exhibited a broad resistance profile with decreased susceptibility to meropenem (Fig. 1C). The no-cost or low fitness cost associated with derepression of the MexAB efflux system (Fig. 4A–C) suggests that these mutants could both be pre-existing in a non-exposed population and will most likely stably remain in the population once the antibiotic pressure is removed [25,26]. The prevalence of isolates that overproduce an efflux pump in nosocomial *P. aeruginosa* strains is estimated to be 60%, and as high as 75% in fluoroquinolone-resistant isolates [23]. Selection for broad-spectrum resistance mechanisms was not only identified during combination therapy, but also with antibiotic concentrations just above the MIC ($1–2 \times$ MIC) the broad-spectrum *mexA*-related resistance could be identified (Supplementary Table S3), indicating large potential problems with dosing and suboptimal treatment regimens [27].

This work showed that treatment with combinations of antimicrobial agents has the potential risk of selecting for multiresistance mechanisms that confer broad-spectrum resistance. We demonstrate that the frequency of pre-existing mutations conferring resistance to two drugs simultaneously was not necessarily lower

than the frequency of mutations after exposure to a single drug and that derepression of the MexAB efflux system is at little or no fitness cost. These results suggested that broad-spectrum resistance could emerge quickly during the use of some combination therapies in clinical settings. Interestingly, mutations in *mexR* and *mexAB* are among the genes most frequently associated with host adaptation in *P. aeruginosa* isolates from CF patients that have frequently undergone several different antibiotic combination therapies [3]. Moreover, recent in vitro investigations in *E. coli* indicate that combination therapy can preferentially select for efflux pump-mediated resistance [28]. In this work, combinations of several different classes of antibiotics preferentially selected for the multiple antibiotic resistance (*mar*) phenotype, indicating that the evolution of broad-range resistance may not only be restricted to ceftazidime + ciprofloxacin combination in *P. aeruginosa* [28]. In addition, extended resistance might be fixed in bacterial populations and difficult to eradicate once the resistance phenotype is present because of the low or non-existent fitness cost. The broad substrate range of many efflux mechanisms that confer resistance against drugs the bacterium was not previously exposed to is particularly worrying [29]. Our observation that multiple mechanisms conferring broad-spectrum antibiotic resistance could be selected with the ceftazidime + ciprofloxacin combination indicates that combination therapy does not necessarily lead to reduced resistance compared with single-drug exposure. We suggest that all antibiotic combinations should be analysed before being implemented in clinical settings (novel and already existing combinations) in order to assess the risk of selecting for broad-spectrum resistance mechanisms. These analyses are necessary in order to choose combination therapies that do not increase the risk of selecting for broad-spectrum resistance mechanisms.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijantimicag.2015.09.014>.

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Competing interests

None declared.

Ethical approval

Not required.

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